Detection of *Actinobacillus pleuropneumoniae* in the Porcine Upper Respiratory Tract as a Complement to Serological Tests

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ABSTRACT

Attempts were made to isolate *Actinobacillus pleuropneumoniae* from the nasal cavities and tonsils of 442 healthy pigs from 15 herds. Samples were streaked onto different media formulations. Serum samples were assayed for antibodies to *A. pleuropneumoniae* by enzyme-linked immunosorbent assay and complement fixation test. *Actinobacillus pleuropneumoniae* was isolated from the nasal cavities only in 24 pigs, from tonsils only in 90 pigs, and from both the nasal cavities and the tonsils in 11 pigs. A PPLO medium supplemented with lincomycin, bacitracin and crystal violet allowed recovery of *A. pleuropneumoniae* from more animals than a tryptic soy agar medium from both sites. Incubation of plates in an enriched CO_2 atmosphere did not affect the recovery rate. *Actinobacillus pleuropneumoniae* belonging to serotypes 1, 2, 3, 5a, 5b, 7, 8, 10 and 12 were isolated, and, in several herds, more than one serotype were recovered. Serotypes of *A. pleuropneumoniae* were isolated from nine herds which were found seronegative to these. The isolation of *A. pleuropneumoniae* from the upper respiratory tract can be useful for detection of carrier pigs and complements serological screening.

RÉSUMÉ

Cette étude portait sur l'isolement d'*Actinobacillus pleuropneumoniae* à partir des cavités nasales et des amygdales de 442 porcs sains dans 15 troupeaux. Des échantillons pris au niveau de ces deux sites ont été ensemencés sur différentes formulations de milieux de culture. Des échantillons de sérum ont été prélevés pour la détection d'anticorps contre *A. pleuropneumoniae* par des épreuves ELISA et de déviation du complément. *Actinobacillus pleuropneumoniae* a été isolé des cavités nasales seulement de 24 porcs, des amygdales seulement de 90 porcs, et simultanément des cavités nasales et des amygdales de 11 porcs. À partir du nez et des amygdales le milieu à base PPLO supplémenté de lincomycine, bacitracine et cristal violet a permis l'isolement d'*A. pleuropneumoniae* de plus d'animaux qu'un milieu à base de TSA. L'incubation des géloses dans une atmosphère enrichie de CO_2 n'a eu aucun effet sur le taux d'isolement. Des isolates d'*A. pleuropneumoniae* appartenant aux sérotypes 1, 2, 3, 5a, 5b, 7, 8, 10, et 12 ont été obtenus, et dans plusieurs troupeaux plus de un sérotype étaient retrouvés. Des sérotypes de *A. pleuropneumoniae* ont été isolés de neuf troupeaux identifiés comme séro-negatifs pour le sérotype retrouvé. L'isolement d'*A. pleuropneumoniae* à partir des voies respiratoires supérieures s'est avéré utile pour la détection d'animaux porteurs asymptomatiques et pourrait être une méthode de diagnostic complémentaire aux tests sérologiques pour identifier les troupeaux infectés chroniquement.

INTRODUCTION

Actinobacillus pleuropneumoniae, a gram-negative bacterium belonging to the family *Pasteurellaceae*, is the causative agent of porcine pleuropneumonia, a severe and contagious respiratory disease of swine (1). The disease is a significant problem in the swine industry throughout the world (2). Due to the economic impact, strict control measures have to be taken to avoid introduction of the agent into noninfected herds. Serological tests, such as the enzyme-linked immunosorbent assay (ELISA) and complement fixation test (CF), are generally used to identify herds with infected animals (3). However, serology has its limitation in detecting infected animals, and serological results are sometimes questioned when there is no evidence of disease in the herd. Nasal cavities and tonsils of healthy pigs are known to harbor *A. pleuropneumoniae* (4). These sites are also heavily colonized by a variety of other bacterial species, hence making the isolation of *A. pleuropneumoniae* difficult on nonselective medium. For this reason, a selective medium would offer great advantage over nonselective media used routinely in diagnostic bacteriology.

The objectives of this study were to evaluate a medium for the isolation of *A. pleuropneumoniae*, to compare two sites in the upper respiratory tract for isolation of *A. pleuropneumoniae* from carrier pigs, and evaluate the usefulness of this method as a complementary diagnostic tool to the serological tests.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

The nasal cavities of 442 market-weight pigs in 15 different herds

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Financial support was provided by the Conseil des recherches en pêche et en agro-alimentaire du Québec (CORPAQ).

Submitted February 9, 1993.

(25 to 30 pigs sampled per herd) were swabbed with a cotton tipped swab (Becton Dickinson Microbiology Systems, Cockeysville, Maryland) and blood samples taken by venipuncture of the jugular vein either on the farm or at the slaughterhouse. Each animal was identified by tattoo and on the same day or on the day following the collection of samples, pigs were sent to the slaughterhouse. After the head had been removed, the tonsils were collected and put into a sterile bag (Canlab, Montréal, Quebec).

Nasal swabs and tonsils were kept in an insulated container with ice-packs and brought to the laboratory within eight hours. Nasal swabs were streaked onto the different media shortly after arrival at the laboratory. Tonsils were stored at −70°C and processed within a month after collection. Sera were stored at −70°C.

CULTURE MEDIA

Two different culture media were used in parallel. The first was made of tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan), with 1 mg/mL NAD (Sigma Chemical Co., St. Louis, Missouri), 1 µg/mL crystal violet (Fisher Scientific Limited, Montréal, Québec), 1 µg/mL lincomycin (Upjohn, Orangeville, Ontario), 6 IU/mL bacitracin (Sigma Chemical Co.) and 5% calf blood (5). The second medium was PPLO agar (Difco Laboratories), with 2 µg/mL of crystal violet, 1 µg/mL of lincomycin, 6 IU/mL of bacitracin, and 1 µg/mL of NAD.

SEROLOGICAL TESTS

All sera were checked for presence of antibodies to *A. pleuropneumoniae* serotypes 1, 2, 5, 3 (cross-reacting with serotypes 6 and 8) and 4 (cross-reacting with serotype 7) using CF test (6) and ELISA as described (3,7,8).

A serum was declared positive if it reacted either with the CF test or the ELISA. A herd was declared positive when one or more pigs were found seropositive.

ISOLATION AND IDENTIFICATION OF *A. PLEUROPNEUMONIAE*

Nasal swabs were streaked onto the TSA medium and two PPLO plates. The TSA medium and one PPLO plate were incubated at 37°C for 18 h in normal atmosphere whereas the second PPLO plate was incubated at 37°C for 18 h in 5% CO_2. Following incubation, plates were examined with a binocular microscope in order to select the suspect colonies. Tonsils were thawed and the surface seared with a hot spatula. A dry cotton swab was used to swab an incision and then inoculate the different media.

On PPLO medium, typical colonies of *A. pleuropneumoniae* were 1 to 2 mm in diameter, smooth and transparent with various degree of iridescence. This latter characteristic was used as a selection criterion, thus only capsulated microorganisms were selected (9). On the TSA medium *A. pleuropneumoniae* suspect colonies were selected on the basis of their enhanced growth around the *Staphylococcus* streak and hemolysis. A maximum of three suspect colonies per plate were picked and transferred onto 5% calf blood agar (CBA), containing 1 mg/mL NAD, to confirm the hemolytic property and for a Gram stain. Suspect hemolytic colonies were tested for their growth factors requirement by inoculating them on a Mueller-Hinton plate to which was added filter paper strips impregnated with NAD. After 24-hour incubation at 37°C in normal atmospheric conditions, all plates were examined for satellite growth around the paper strip. Urease production was verified by streaking a loopful of bacteria on slant of tryptic soy agar containing 0.1 mg/mL urea, 1 mg/mL NAD and phenol red. The Christie, Atkins and Munch-Peterson (CAMP) reaction was tested by streaking suspect isolates perpendicular to β-toxin producing *S. aureus* streaked on CBA plates. A positive reaction was indicated by an enhanced zone of hemolysis in the incomplete β-toxin zone around the *Actinobacillus* streak. By using the aforementioned selection criteria, only typical capsulated *A. pleuropneumoniae* isolates were selected and nonhemolytic as well as NAD-independent isolates were not picked up.

TABLE I. Number of pigs positive for isolation of *Actinobacillus pleuropneumoniae* from two sampling sites using two different culture media

Sites	Number of pigs sampled	Number of positive pigs: PPLO base medium, Without CO_2 enrichment	PPLO base medium, With CO_2 enrichment	TSA base medium	Total
Nasal cavities	442	13	14	13	35[b]
Tonsils	429	46	50	22	101[b]
Total	...	55[a]	61[a]	30[a]	

[a]Some pigs were culture positive at both sites and by different or more than one culture procedure
[b]Includes 11 pigs that were culture positive from the nose and tonsils simultaneously

SEROTYPING

All isolates identified as *A. pleuropneumoniae* were serotyped by coagglutination (10), immunodiffusion and counter-immunoelectrophoresis (11). The indirect hemagglutination test was used to differentiate cross-reacting strains (12).

RESULTS

Table I shows the results of the isolation of *A. pleuropneumoniae* under the different culture conditions from the two sites sampled. When the nasal cavities were sampled, the PPLO base medium incubated under normal atmosphere allowed recovery of *A. pleuropneumoniae* from the same number of animals, but not necessarily the same animals, as the TSA medium. When a CO_2 enriched atmosphere was used with the PPLO base, 14 pigs were found positive, nine of which were negative under normal atmosphere.

When the tonsils were cultured, more than twice as many *A. pleuropneumoniae* positive sites were obtained with PPLO as with TSA medium. Of the 50 positive tonsils obtained under CO_2 enriched atmosphere, only 27 were also positive under normal atmosphere incubation. Of the 442 pigs which were sampled, *A. pleuropneumoniae* was recovered from the nasal cavities only in 24 pigs, from the tonsils only in 90 pigs and from both sites in 11 pigs.

TABLE II. Serotypes of *Actinobacillus pleuropneumoniae* isolated from two sampling sites in 15 herds

Herd	No. pigs sampled Nose/tonsil	No. pigs positive	Serotypes								
			1	2	3	5a	5b	7	8	10	12
A	25/25	3	1/3[a]	··/··	··/··	··/··	··/··	··/··	··/··	··/··	··/··
B	30/30	7	··/··	··/··	··/1	1/2	··/··	1/1	··/··	··/··	··/1
C	30/30	8	··/3	··/··	··/··	··/··	··/··	··/1	··/··	··/··	··/5
D	30/30	16	··/1	··/··	··/1	··/··	2/9	1/4	··/1	··/··	··/2
E	30/30	15	··/··	··/1	··/1	··/··	··/3	··/8	··/··	··/··	··/3
F	27/25	9	··/1	··/··	··/··	··/··	··/4	2/1	··/··	··/··	2/··
G	30/30	9	1/2	··/··	··/··	··/··	··/··	2/1	··/··	1/··	1/1
H	30/29	16	··/··	··/··	··/··	··/··	··/··	6/12	··/··	··/··	1/··
I	30/29	4	··/··	··/··	··/··	··/··	··/3	··/1	··/··	··/··	··/··
J	30/30	6	··/··	··/··	··/··	2/5	··/··	··/··	··/··	··/··	··/··
K	30/27	1	··/··	··/··	··/··	··/··	··/1	··/··	··/··	··/··	··/··
L	30/27	7	··/··	··/··	··/··	··/··	··/··	2/··	··/··	··/··	··/5
M	30/30	7	··/··	3/3	··/1	··/··	··/··	··/··	··/··	··/··	··/··
N	30/30	9	··/··	··/··	··/6	··/··	3/2	··/··	··/··	··/··	··/··
O	30/29	8	··/··	··/··	··/··	··/··	2/2	··/··	··/3	1/··	··/1

[a]Number of positive pigs at each site

TABLE III. Serological status, on an individual basis, and serotypes of *Actinobacillus pleuropneumoniae* isolated

Herd	Number of pigs tested	Serology						Serotype isolated
		1	2	3,6,8[a]	5[b]	4,7[a]	10,12	
A	25	11[c]	··	··	··	··	N. D.[d]	1
B	30	··	2	12	11	3	N. D.	3, 5a, 7, 12
C	30	3	4	··	··	2	N. D.	1, 7, 12
D	30	··	5	2	10	5	N. D.	1, 3, 5b, 7, 8, 12
E	30	··	··	2	11	8	N. D.	2, 3, 5b, 7, 12
F	27	··	1	··	13	15	N. D.	1, 5b, 7, 12
G	30	··	··	10	··	1	N. D.	1, 7, 10, 12
H	30	··	1	··	1	30	N. D.	7, 12
I	30	··	··	5	10	9	N. D.	5b, 7
J	30	··	3	3	19	3	N. D.	5a
K	30	··	··	··	3	··	N. D.	5b
L	30	··	··	1	1	18	N. D.	7, 12
M	30	3	1	··	1	2	N. D.	2, 3
N	30	··	5	30	19	7	N. D.	3, 5b, 12
O	30	··	4	3	4	3	N. D.	5b, 8, 10, 12

[a]These serotypes are grouped together because the antigen used in the serological tests cannot distinguish reactions due to the specific serotype
[b]Includes serotypes 5a and 5b
[c]Number of seropositive pigs
[d]Not done

Table II shows the distribution of the different serotypes in each herd. The most prevalent serotype in the nasal cavities was serotype 7 with 12 positive pigs including six pigs from one herd. As noted with the isolates from the nasal cavities, a number of different serotypes were found in the tonsils. In each of five pigs, two different serotypes were obtained from the tonsils. Serotype 7 was again the most prevalent with 27 positive pigs, including 12 pigs from the same herd that had six pigs positive for culture from the nasal cavities. In only two of those 12 pigs was *A. pleuropneumoniae* serotype 7 isolated from both the nasal cavities and tonsils of the same animal. Isolation of *A. pleuropneumoniae* from the nasal cavities and tonsils of the same animal occurred in only 11 pigs, and in five of these 11 pigs, different serotypes were isolated from the two sites. All but three herds (A, J and K) were harboring more than one serotype of *A. pleuropneumoniae*. In the herds that had more than one serotype isolated, no apparent pattern of association of serotypes were noted. It is noteworthy that, although the number of positive pigs is low, serotypes 3 and 8 were recovered only from the tonsils and serotype 10 only from the nasal cavities.

Results on the detection of antibodies to *A. pleuropneumoniae* are presented in Table III. In addition, the serotypes isolated (from the nasal cavities and from the tonsils) in each herd are shown. The same three herds (A, J and K) that were culture positive for only one serotype were seropositive to only one serotype; all other herds were seropositive to more than one serotype and in fact most of the herds were seropositive to three or four serotypes.

To assess the correlation between the serological results and the isolation of *A. pleuropneumoniae* on a herd basis, for each serotype or known cross-reacting serotypes the status of the 15 herds based on serology and isolation were tabulated (Table IV).

DISCUSSION

One of the main concerns of swine producers is to prevent entry of an infectious disease into their herd through the introduction of infected animals. Porcine pleuropneumonia is one of the most undesirable diseases that can become established in a herd. When clinical signs and pathological examination suggest the presence of the disease, isolation of *A. pleuropneumoniae* from the lungs generally confirms the diagnosis. In other circumstances, the serological analysis of a representative number of sera from a herd by ELISA and CF test can generally establish if the herd had been exposed to the bacterium, but in the absence of clinical signs and lung lesions the reliability of the serological tests is often questioned.

The 15 herds in this study had previous serological indications of contact with *A. pleuropneumoniae* but had no clinical or pathological cases at the time of sampling. Sampling of the nasal cavities and tonsils made it possible to isolate *A. pleuropneumoniae* in each of the 15 herds and concomittant serological test results still suggested the presence of the agent. The PPLO selective medium allowed the highest recovery rate of *A. pleuropneumoniae*. Previous investigators (5) had indicated that a selective medium for isolating the agent of porcine pleuropneumonia was most useful with samples from the upper respiratory tract. A nonselective medium (4) has also been used in the present investigation (results not shown) but it is much more laborious because the isolates must rapidly (≤6 h) produce capsular material in order to detect the iridescence necessary to select the suspect colonies. This latter characteristic was used in the present investigation and combined with the advantages of a selective medium.

In this study, the overall isolation rate of *A. pleuropneumoniae* from the nasal cavities (7.9%) is slightly higher than the one found (4.4%) by Gilbride and Rosendal (13) in market-weight pigs. It is, however, much lower than the one reported by Kume *et al* (4) who found that 47.3% of 619 pigs harbored *A. pleuropneumoniae* in the nasal cavity. In the latter case, it appears that the pigs originated from herds infected only with serotype 2. Gilbride and Rosendal (5) had found serotype 7 isolates of *A. pleuropneumoniae* as well as some untypable isolates. In a herd known to be endemically infected with serotype 5, the highest isolation rate from the nasal cavities was 30% in 12-week-old pigs (14). All the isolates of *A. pleuropneumoniae* recovered from the nasal cavities in the present investigation were serotyped and belonged to seven different serotypes (including 5a and 5b).

The tonsils proved to be a better site than the nasal cavities for isolation of *A. pleuropneumoniae* with as many as almost three times more positive pigs (23.5% vs. 7.9% of the animals sampled). The increased isolation rate from the tonsils was particularly evident when the PPLO base medium was used. Even so, the best culture conditions (PPLO medium and CO_2 enrichment) taken individually would have only allowed detection of 50% of carrier animals. Results of Table I are indicative that the opportunity of isolating *A. pleuropneumoniae* from carrier animals increases with the number of attempts to isolate this bacterium; in this study, six plates per pigs were used and the number of culture positive pigs was 125 or 29% of animals sampled at both sites.

TABLE IV. Correlation between the serological status and the presence of *Actinobacillus pleuropneumoniae* in the herds

Herd status based on isolation	Herd status based on serology: 1		2		3,6,8[a]		5		4,7[a]	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Positive	2[b]	3	1	1	3	3	9	0	7	2
Negative	0	10	3	10	3	9	0	6	2	4

[a]These serotypes are grouped together because the antigen used in the serological tests cannot distinguish reactions due to the specific serotype
[b]Number of herds

The distribution pattern of the serotypes isolated from the nasal cavities and tonsils is somewhat more uniform than the one noted for the strains isolated over the last decade in Québec from the lungs of pigs with clinical pleuropneumonia. In those cases 68% of the isolates belonged to serotype 1, 23% to serotype 5 and 9% to serotypes 2, 3, 6, 7, 8, 10 and 12 together (15). It is possible that the isolates found in the present investigation, even those of serotypes 1 and 5, are less virulent than those obtained from the lungs of diseased pigs and consequently are unable to cause clinical disease by establishing themselves beyond the upper respiratory tract. The presence of multiple serotypes in different animals, and even in the same animal, shows the complexity of the laboratory diagnosis of this infection.

The use of a CO_2 enriched atmosphere did not increase the rate of isolation of *A. pleuropneumoniae* over that obtained with a normal atmosphere. Less than 40% of the isolates were obtained from the same pigs when the two atmospheric conditions were compared; it is not known if this reflects possible difference between the growth characteristics of some of the isolates, difficulty in recognizing the isolate on the plates or paucity of the organisms on the plates. It is thus suggested to use both conditions when attempting to isolate *A. pleuropneumoniae* from healthy carriers or to inoculate more than one plate per animal sampled.

In all 15 herds serological evidence of infection was confirmed by isolation of *A. pleuropneumoniae*. Kume *et al* (4) were able to isolate *A. pleuropneumoniae* from 92% of one to 16-week-old pigs housed on conventional farms and from 90% of four-month-old pigs housed on parent and grandparent stock farms that had CF antibody titers >4. Serotype 2 was the only serotype found by culture and the only one to which the animals had detectable CF antibodies. Wilson *et al* (14) could establish a correlation between isolation and ELISA serology results with *A. pleuropneumoniae* serotype 5 when the antibody titer was greater than 50% of the positive control value. In this study, an attempt to correlate isolation and serology was done only on a herd basis, because isolation is not a sensitive diagnostic tool for individual pigs and serology has limited sensitivity on an individual basis. *Actinobacillus pleuropneumoniae* serotypes 4, 9 and 11 have never previously been demonstrated in Québec and serology cannot differentiate between infection by serotypes 1 and 9, 4 and 7 and 3, 6 and 8. We did not include serotypes 10 and 12 in our serology since they have been isolated only occasionally in Québec.

We noted that of the 31 culture positive herds, nine were seronegative for a serotype found by isolation; except for serotype 5 where serology and isolation correlated. This observation indicates that the modified ELISA test is highly specific for serotype 5 (7). On the other hand, we are concerned about serology not detecting herds that are positive by isolation, especially in the case of serotype 1 strains which include the most virulent strains of *A. pleuropneumoniae* in Québec. The fact that several of the herds were infected with more than one serotype could have affected the serological response of pigs to other serotypes, or false negative reactions could be due to an improper threshold use in the ELISA test. Finally, recognizing the lack of sensitivity of isolation procedure to detect infected pigs, one has to wonder if the seropositive herds which did not yield an isolate belonging to the homologous serotype might be due to minor cross-reactions with other serotypes.

In conclusion, this study has shown that the PPLO medium with selective agents is advantageous for the isolation of *A. pleuropneumoniae* from chronically infected pigs. Furthermore, the use of multiple plates and atmospheres with and without increased CO_2 will increase the recovery of the microorganism. Finally, the tonsils appear to be a better site for isolation of *A. pleuropneumoniae* than the nasal cavities. This study has clearly demonstrated the need to use both isolation and serology for the detection of chronically infected herds.

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